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(54) Title: DOUBLE MUTANT ENTEROTOXIN FOR USE AS AN ADJUVANT			
(57) Abstract			
<p>The present invention is directed towards a novel composition which is a genetically distinct mutant of <i>E. coli</i> heat-labile enterotoxin (LT). Specifically, the mutant LT designated LT(R192G/L211A) is modified by two amino acid substitutions, i.e., the arginine at amino acid position 192 is replaced by glycine and the leucine at amino acid position 211 is replaced by alanine. The invention relates to compositions and methods for use of the novel double mutant of LT as an adjuvant.</p>			

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DOUBLE MUTANT ENTEROTOXIN FOR USE AS AN ADJUVANT**1. FIELD OF THE INVENTION**

The present invention is directed towards a novel
5 composition which is a genetically distinct mutant of *E. coli*
heat-labile enterotoxin (LT). Specifically, the mutant LT
designated LT(R192G/L211A) is modified by two amino acid
substitutions, i.e., the arginine at amino acid position 192
is replaced by glycine and the leucine at amino acid position
10 211 is replaced by alanine. The invention relates to
compositions and methods for use of the novel double mutant
of LT as an adjuvant.

2. BACKGROUND OF THE INVENTION

The World Health Organization report of Infectious
15 Disease deaths for 1995 indicated that there were more than
13 million deaths world-wide during that year. The majority
of those deaths were caused by organisms that first make
contact with and then either colonize or cross mucosal
surfaces to infect the host. The overall morbidity caused by
20 these organisms and other pathogens that interact with
mucosal surfaces is impossible to calculate.

Traditional vaccine strategies that involve
parenteral immunization with inactivated viruses or bacteria
or subunits of relevant virulence determinants of those
25 pathogens do not prevent those interactions. In fact,
traditional vaccine strategies do not prevent infection but
instead resolve infection before disease ensues. In some
cases, HIV for example, once the virus crosses the mucosal
surface and enters the host cell, be that a dendritic cell,
an epithelial cell, or a T-cell, the host-parasite
30 relationship is moved decidedly in favor of the parasite
(HIV). In that case, as in many others, a vaccine strategy

that does not prevent the initial infection of the host is unlikely to succeed.

Recently, a great deal of attention has focused on mucosal immunization as a means of inducing secretory IgA (sIgA) antibodies directed against specific pathogens of mucosal surfaces. The rationale for this is the recognition that sIgA constitutes greater than 80% of all antibodies produced in mucosal-associated lymphoid tissues in humans and that sIgA may block attachment of bacteria and viruses, neutralize bacterial toxins, and even inactivate invading viruses inside of epithelial cells. In addition, the existence of a Common Mucosal Immune System permits immunization on or at one mucosal surface to induce secretion of antigen-specific sIgA at distant mucosal sites. It is only now being appreciated that mucosal immunization may be an effective means of inducing not only sIgA but also systemic antibody and cell-mediated immunity.

The mucosal immune response can be divided into two phases (McGhee and Kiyono, 1993, *Infect Agents Dis* 12:55-73). First, the inductive phase involves antigen presentation and the initiation events which dictate the subsequent immune response. During the initiation events, antigen-specific lymphocytes are primed and migrate from the inductive sites (e.g., Peyer's patches in the enteric mucosa) through the regional lymph nodes, into the circulation and back to mucosal effector sites (e.g., lamina propria). Once these effector cells have seeded their effector sites, the second phase, or effector phase, of the mucosal immune response can occur. A significant difference between mucosal immunization and parenteral immunization is that both mucosal and systemic immunity can be induced by mucosal immunization while parenteral immunization generally results only in systemic responses.

Most studies conducted to date have dealt with the secretory antibody component of the mucosal response and the complex regulatory issues involved with induction of sIgA following mucosal immunization and not with the systemic antibody response or cellular immunity induced by mucosal immunization. In that regard, it is important to understand the type of helper T lymphocyte response induced by mucosal immunization since the type of helper T lymphocyte stimulated by an antigen is one of the most important factors for defining which type of immune response will follow. Mosmann and colleagues (Cherwinski et al., 1987, *Journal of Experimental Medicine* 166:1229-1244; Mosmann and Coffman, 1989, *Annual Reviews of Immunology* 7:145-173) discovered that there are at least two different types of helper T lymphocytes (Th) which can be identified based on cytokine secretion. Th1 lymphocytes secrete substantial amounts of IL-2 and INF-gamma and execute cell-mediated immune responses (e.g., delayed type hypersensitivity and macrophage activation), whereas Th2 lymphocytes secrete IL-4, IL-5, IL-6 and IL-10 and assist in antibody production for humoral immunity. Theoretically then, antigenic stimulation of one T helper cell subset and not the other would result in production of a particular set of cytokines which would define the resulting immune response.

The presence of IL-2 and INF-gamma coupled with an antigenic stimulus presented by macrophages in the context of Class II MHC molecules can initiate Th1 type responses. The ability of Th1 cells to secrete IL-2 and INF-gamma further amplifies the response by activating Th1 cells in an autocrine fashion and macrophages in a paracrine fashion. These activated leukocytes can release additional cytokines (e.g., IL-6) which may induce the proliferation and differentiation of antigen specific B lymphocytes to secrete antibody (the effector phase). In this scenario, the

predominant isotype secreted by murine B lymphocytes is often IgG2a. In a second scenario (Urban et al., 1992, *Immunol Rev* 127:205-220), antigens such as allergens or parasites can effectively stimulate a Th2 lymphocyte response (the inductive phase). Presentation of such antigens to Th2 cells can result in the production of the lymphokines IL-4 and IL-5 which can induce antigen specific B lymphocytes to secrete IgE and IgG1 or induce eosinophilia, respectively (the effector phase). Furthermore, stimulated Th2 cells can secrete IL-10 which has the ability to specifically inhibit secretion of IL-2 and INF-gamma by Th1 lymphocytes and also to inhibit macrophage function.

While these representations are simplistic, it is obvious that the type of T helper cell stimulated affects the resultant cellular immune response as well as the predominant immunoglobulin isotype secreted. Specifically, IL-4 stimulates switching to the IgE and IgG1 isotypes whereas INF-gamma stimulates IgG2a secretion. Numerous studies, predominantly conducted in vitro, have suggested that IL-5, IL-6 and TGF-beta (Th3) can cause isotype switching to IgA.

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2.1. MUCOSAL ADJUVANTS

Mucosally administered antigens are frequently not immunogenic. A number of strategies have been developed to facilitate mucosal immunization, including the use of attenuated mutants of bacteria (e.g., *Salmonella* spp.) as carriers of heterologous antigens, encapsulation of antigens into microspheres, gelatin capsules, different formulations of liposomes, adsorption onto nanoparticles, use of lipophilic immune stimulating complexes, and addition of bacterial products with known adjuvant properties. The two bacterial products with the greatest potential to function as mucosal adjuvants are cholera toxin (CT), produced by various strains of *Vibrio cholerae*, and the heat-labile enterotoxin

(LT) produced by some enterotoxigenic strains of *Escherichia coli* (Clements et al., 1988, *Vaccine* 6:269-277; Elson, 1989, *Immunology Today* 146:29-33; Lycke et al., 1992, *European Journal of Immunology* 22:2277-2281; Xu-Amano et al., 1993, *Journal of Experimental Medicine* 178:1309-1320; Yamamoto et al., 1996, *Annals of the New York Academy of Sciences* 778:64-71).

Although LT and CT have many features in common, these are clearly distinct molecules with biochemical and immunologic differences which make them unique (see below). Both LT and CT are synthesized as multisubunit toxins with A and B components. On thiol reduction, the A component dissociates into two smaller polypeptide chains. One of these, the A1 piece, catalyzes the ADP-ribosylation of the stimulatory GTP-binding protein (GSa) in the adenylate cyclase enzyme complex on the basolateral surface of the epithelial cell resulting in increasing intracellular levels of cAMP. The resulting increase in cAMP causes secretion of water and electrolytes into the small intestine through interaction with two cAMP-sensitive ion transport mechanisms involving 1) NaCl cotransport across the brush border of villous epithelial cells, and 2) electrogenic Na dependent Cl secretion by crypt cells (Field, 1980, *Secretory Diarrhea* pp21-30). The B-subunit binds to the host cell membrane receptor (ganglioside GM1) and facilitates the translocation of the A-subunit through the cell membrane.

Recent studies have examined the potential of CT and LT as a mucosal adjuvant against a variety of bacterial and viral pathogens using whole killed organisms or purified subunits of relevant virulence determinants from these organisms. Representative examples include tetanus toxoid (Xu-Amano et al., 1993, *Journal of Experimental Medicine* 178:1309-1320; Yamamoto et al., 1996, *Annals of the New York Academy of Sciences* 778:64-71; Xu-Amano et al., 1994, *Vaccine*

12:903-911), inactivated influenza virus (Hashigucci et al., 1996, *Vaccine* 14:113-119; Katz et al., 1996, *Options for the control of influenza. III.*, pp292-297; Katz et al., 1997, *Journal of Infectious Diseases* 175:352-363), recombinant
5 urease from *Helicobacter spp.* (Lee et al., 1995, *Journal of Infectious Diseases* 172:161-171; Weltzin et al., 1997, *Vaccine* 4:370-376), pneumococcal surface protein A from *Streptococcus pneumoniae* (Wu et al., 1997, *Journal of Infectious Diseases* 175:839-846), Norwalk virus capsid
10 protein, synthetic peptides from measles virus (Hathaway et al., 1995, *Vaccine* 13:1495-1500), and the HIV-1 C4/V3 peptide T1SP10 MN(A) (Staats et al., 1996, *Journal of Immunology* 157:462-472). There are many other examples and it is clear that both LT and CT have significant potential for use as adjuvants for mucosally administered antigens (see Dickinson
15 and Clements, 1996, *Mucosal Vaccines* pp.73-87 for a recent review). This raises the possibility of an effective immunization program against a variety of pathogens involving the mucosal administration of killed or attenuated agents or relevant virulence determinants of specific agents in
20 conjunction with LT or CT. However, the fact that these "toxins" can stimulate a net luminal secretory response may prevent their use. For instance, as little as 5 μ g of purified CT was sufficient to induce significant diarrhea in volunteers while 25 μ g was shown to elicit a full 20-liter
25 cholera purge (Levine et al., 1983, *Microbiological Reviews* 47:510-550). In recently conducted volunteer studies with LT administered alone or in conjunction with the *V. cholerae* Whole Cell/B-Subunit Vaccine, LT was shown to induce fluid secretion at doses as low as 2.5 μ g when administered in
30 conjunction with the vaccine, while 25 μ g of LT elicited up to 6-liters of fluid. While the adjuvant effective dose in humans for either of these toxins has not been established, experiments in animals suggest that it may be a comparable to

the toxic dose. Taken together, these studies indicate that while LT and CT may be attractive as mucosal adjuvants, studies in animals do not reflect the full toxic potential of these molecules in humans, and that toxicity will seriously
5 limit their practical use for humans.

A number of attempts have been made to alter the toxicity of LT and CT, most of which have focused on eliminating enzymatic activity of the A-subunit associated with enterotoxicity. The majority of these efforts have involved the use of site-directed mutagenesis to change amino
10 acids associated with the crevice where NAD binding and catalysis is thought to occur. Recently, a model for NAD binding and catalysis was proposed (Domenighini et al., 1994, *Molecular Microbiology* 14:41-50; Pizza et al., 1994, *Molecular Microbiology* 14:51-60) based on computer analysis
15 of the crystallographic structure of LT (Sixma et al., 1991, *Nature (London)* 351:371-377; Sixma et al., 1993, *Journal of Molecular Biology* 230:890-918). Replacement of any amino acid in CT or LT involved in NAD-binding and catalysis by site-directed mutagenesis has been shown to alter
20 ADP-ribosyltransferase activity with a corresponding loss of toxicity in a variety of biological assay systems (Lycke et al., 1992, *European Journal of Immunology* 22:2277-2281; Burnette et al., 1991, *Infection and Immunity* 59:4266-4270; Harford et al., 1989, *European Journal of Biochemistry* 183:311-316; Häse et al., 1994, *Infection and Immunity* 62:3051-3057; Lobet et al., 1991, *Infection and Immunity* 59:2870-2879; Merritt et al., 1995, *Nature Structural Biology* 2:269-272; Moss et al., 1993, *Journal of Biological Chemistry* 268:6383-6387; Tsuji et al., 1991, *FEBS Letters* 291:319-321; Tsuji et al., 1990, *Journal of Biological Chemistry* 265:22520-22525).
25
30 In addition, it has been shown that exchanging K for E112 in LT not only removes ADP-ribosylating enzymatic activity, but cAMP activation and adjuvant activity

as well (Lycke et al., 1992, *European Journal of Immunology* 22:2277-2281). A logical conclusion from the Lycke et al. studies was that ADP-ribosylation and induction of cAMP are essential for the adjuvant activity of these molecules. As a result, a causal linkage was established between adjuvant activity and enterotoxicity. That is, the accumulation of cAMP responsible for net ion and fluid secretion into the gut lumen was thought to be a requisite to adjuvant activity. Recent studies by a number of laboratories have challenged that linkage.

Dickinson and Clements (Dickinson and Clements, 1995, *Infection and Immunity* 63:1617-1623) (Clements et al.) explored an alternate approach to dissociation of enterotoxicity from adjuvant activity. LT requires proteolysis of a trypsin sensitive bond to become fully active. In this enterotoxin, that trypsin sensitive peptide is subtended by a disulfide interchange that joins the A1 and A2 pieces of the A-subunit. In theory, if the A1 and A2 pieces cannot separate, A1 will not be able to find its target (adenylate cyclase) on the basolateral surface or assume the conformation necessary to bind or hydrolyze NAD.

The mutant of Clements et al. has been described more fully in PCT Publication WO96/06627, incorporated herein by reference. The mutant LT holotoxin, designated LT(R192G), was constructed using site-directed mutagenesis to create a single amino acid substitution within the disulfide subtended region of the A-subunit separating A1 from A2. This single amino acid change altered the proteolytically sensitive site within this region, rendering the mutant insensitive to trypsin activation. The physical characteristics of this mutant were examined by SDS-PAGE, its biological activity was examined on mouse Y-1 adrenal tumor cells and Caco-2 cells, its enzymatic properties determined in an in vitro NAD:agmatine ADP-ribosyltransferase assay, and its

immunogenicity and immunomodulating capabilities determined by testing for the retention of immunogenicity and adjuvanticity.

PROPERTIES OF LT(R192G)

- 5 • 100 - 1,000 fold less active than cholera toxin or native LT in the mouse Y-1 adrenal cell assay
- Not sensitive to proteolytic activation
- Does not possess in vitro NAD:agmatine ADP-ribosyltransferase activity
- 10 • Does not increase production of cAMP in cultured Caco-2 cells
- Reduced enterotoxicity in the patent mouse intestinal challenge model when compared to native LT
- Promotes the development of both humoral (antibody) and cell-mediated immune responses against co-
- 15 administered antigens of a pathogenic microorganism in both the systemic and mucosal compartments
- Functions as an effective adjuvant when administered mucosally (i.e., orally, intranasally) or parenterally (i.e., subcutaneously)
- 20 • Lacks enterotoxicity in humans at adjuvant-effective doses

LT(R192G) has been shown to possess the capability of enhancing an immune response (e.g., IgG, IgA) to antigens unrelated to LT or LT(R192G). Recent experimental evidence

25 shows that LT(R192G) has utility as an adjuvant for mucosally or parenterally administered antigens; such administration results in the production of serum IgG and/or mucosal sIgA as well as cell-mediated immune responses against the antigen with which LT(R192G) is delivered and, more importantly, to

30 protect against subsequent challenge with infectious organisms. LT(R192G) has been shown to be an effective

mucosal adjuvant and has recently been evaluated in humans in several Phase I safety studies.

More recently, Tsuji et al. (Tsuji et al., 1997, *Immunology* 90:176-182) demonstrated that a protease-site deletion mutant LT(Δ 192-194) also lacks in vitro
5 ADP-ribosylagmatine activity, has a ten-fold reduction in enterotoxicity in rabbit ligated ileal loops, and a 50% reduction and delayed onset of cAMP induction in cultured myeloma cells. LT(Δ 192-194) was shown to have increased
10 adjuvant activity for induction of serum IgG and mucosal IgA against measles virus when compared to native LT, LT-B, or LT(E112K). LT(Δ 192-194) was effective when administered intranasally, subcutaneously, intraperitoneally, or orally although mucosal IgA responses were only demonstrated
15 following mucosal administration. These investigators also demonstrated increased adjuvant activity for mucosally administered LT(Δ 192-194) in conjunction with KLH, BCG, and Ova. These findings are consistent with the findings with LT(R192G).

20 3. SUMMARY OF THE INVENTION

The present invention provides a new mutant LT molecule containing one additional mutation of leucine at position 211 to alanine (L211A).

More particularly, the present invention provides a novel LT molecule, LT(R192G/L211A), which exhibits the
25 adjuvanticity of LT(R192G), but which surprisingly has even lower enterotoxicity compared to a large dose of LT(R192G) holotoxin. See, for example, Figure 4. It has been noted that 250 μ g of LT(R192G) has less enterotoxicity in the patent mouse assay than does 5 μ g of native LT. The novel
30 double mutant LT(R192G/L211A) has even less enterotoxicity than does LT(R192G) in the patent mouse assay.

Also provided are methods of using LT(R192G/L211A) as an adjuvant for the stimulation of an immune response against an antigen administered in combination with the novel adjuvant.

5 LT(R192G) has previously been shown to possess the capability of enhancing an immune response (e.g., IgG, IgA) to antigens unrelated to LT or LT(R192G). Recent experimental evidence shows that LT(R192G) has utility as an adjuvant for mucosally or parenterally administered antigens; such
10 administration results in the production of serum IgG and/or mucosal sIgA as well as cell-mediated immune responses against the antigen with which LT(R192G) is delivered and, more importantly, to protect against subsequent challenge with infectious organisms.

It has been surprisingly discovered that modifying
15 the leucine at position 211 of LT(R192G) to alanine results in even lower enterotoxicity than LT(R192G), as measured in the patent mouse assay (see Figure 4). The new molecule, referred to as LT(R192G/L211A), retains the adjuvanticity of LT(R192G), but exhibits even lower enterotoxicity, in the
20 patent mouse assay, than does LT(R192G).

The invention also provides a composition useful in the methods to stimulate an immune response. The composition comprises an effective amount of LT(R192G/L211A) in combination with an effective amount of antigen.

25 4. BRIEF DESCRIPTION OF THE FIGURES

The present invention may be understood more fully by reference to the following detailed description of the invention, examples of specific embodiments of the invention and the appended figures in which:

30 Figure 1 is a schematic diagram of plasmid pCS95, which encodes both subunits LT A and B under the control of the lac promoter. Figure 1A illustrates the construction of

plasmid pCS95 which contains the nucleotide sequence encoding mutant LT(R192G). Plasmid pCS95 was constructed by replacing the BamHI-XbaI of pBD95 with the BamHI-XbaI fragment of pDF82. The lower half of Figure 1B shows the single amino acid change in LT(R192G), encoded for by pCS95. The upper half of Figure 1B shows the additional amino acid change in LT(R192G), which yields plasmid pECD401 coding for LT(R192G/L211A). Plasmid pECD401 provides LT(R192G/L211A) which contains two base substitutions, one at amino acid residue 192 of subunit A, coding for Gly rather than Arg, and another at amino acid residue 211 of subunit A, coding for Ala rather than Leu. All mutations were induced by site directed mutagenesis.

Figure 2 shows a portion of the sequence of LT in the A-subunit, including a portion distal to the trypsin cleavage site at position 192 including the leucine at position 211.

Figure 3A and figure 3B show the sensitivity of wild-type LT and several LT mutants to trypsin and pepsin proteolysis.

Figure 4 shows a patent mouse intestinal assay demonstrating that LT(R192G/L211A) has lower toxicity than an equivalent amount of LT(R192G). For these studies, LT(R192G) and LT(R192G/L211A) were examined for enterotoxicity in the patent mouse assay. Groups of mice were orally inoculated with native LT at 5, 25, or 125 μ g, or with LT(R192G/L211A) at 125 or 250 μ g, or with LT(R192G) at 25, 125, or 250 μ g. Following a three hour interval, the gut:carcass ratio of each animal was determined. The gut-carcass ratio is defined as the intestinal weight divided by the remaining carcass weight. There were three animals per group and the means for each data point are shown.

Figure 5 demonstrates that LT(R192G/L211A) functions equally as well as LT(R192G) as an immunologic

adjuvant for induction of serum IgG when administered intranasally. Mice were immunized intranasally with Ovalbumin (Ova) alone or in conjunction with 5 μ g of LT(R192G/L211A), LT(R192G), or native LT. Serum anti-Ova IgG was determined
5 by ELISA. There were seven animals per group and the means for each data point are shown. Mice immunized intranasally with Ova in conjunction with LT(R192G) had serum anti-Ova IgG responses indistinguishable from animals immunized with Ova in conjunction with LT(R192G/L211A) or in conjunction with native LT.

10 Figure 6 is an experimental demonstration that LT(R192G/L211A) has the same ability as LT(R192G) to function as an immunologic adjuvant for production of antigen-specific Th2-type cytokines, specifically, IL-10, by mononuclear cells from the spleens of animals immunized intranasally. Mice
15 were immunized intranasally with Ovalbumin (Ova) alone or in conjunction with 5 μ g of LT(R192G) or LT(R192G/L211A). Cytokines were determined by ELISA following a T-cell restimulation assay. Mice immunized intranasally with Ova in conjunction with LT(R192G) had Ova-specific IL-10 responses
20 indistinguishable from animals immunized with Ova in conjunction with LT(R192G/L211A).

Figure 7 is an experimental demonstration that LT(R192G/L211A) has the same ability as LT(R192G) to function as an immunologic adjuvant for production of antigen-specific Th2-type cytokines, specifically, IFN-gamma, by mononuclear
25 cells from the spleens of animals immunized intranasally. Mice were immunized intranasally with Ovalbumin (Ova) alone or in conjunction with 5 μ g of LT(R192G) or LT(R192G/L211A). Cytokines were determined by ELISA following a T-cell restimulation assay. Mice immunized intranasally with Ova in
30 conjunction with LT(R192G) had Ova-specific IFN-gamma responses indistinguishable from animals immunized with Ova in conjunction with LT(R192G/L211A).

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a new LT molecule containing a glycine at position 192 in place of an arginine (R192G) and an additional mutation of leucine at position 211 to alanine (L211A) of the A subunit of LT and compositions thereof, and methods of using said compositions as an adjuvant.

The mutant LT(R192G) is shown to have reduced enterotoxicity in the patent mouse assay when compared to native LT (see Figure 4). In a randomized, placebo-controlled, dose escalating study in adult volunteers, 0 of 24 volunteers showed adverse reactions to single oral doses of 2, 25, or 50 μ g of LT(R192G). (Oplinger ML, Baqar S, Trofa AF, Clements JD, Gibbs P, Pazzaglia G, Bourgeois AL, Scott DA, 1997, Safety and Immunogenicity in Volunteers of a New Candidate Oral Mucosal Adjuvant, LT(R192G), Abstr. G-10, p. 193, In: Program and Abstracts of the 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, American Society for Microbiology, Washington, D.C.). This is in contrast to native LT, which was shown to be enterotoxic at 2.5 μ g in volunteers when combined with the B-subunit/Whole Cell Cholera vaccine. However, at 100 μ g of LT(R192G), 2 of 12 (16.7%) of volunteers developed mild to moderate diarrhea which resolved within 24 hours.

The attenuating mutation in LT(R192G) is in the site where proteolytic activation by trypsin-like proteases is thought to occur in the native molecule; that is, the trypsin sensitive Arg residue within the region subtended by the disulfide loop of the A-subunit. Trypsin is not the only protease found in the mammalian intestinal tract. Pepsin is the principle proteolytic enzyme in gastric juice and functions optimally at acidic pH. Pepsin cleaves preferentially at peptide bonds involving the carboxylic groups of aromatic amino acids and other hydrophobic amino

acids (especially phenylalanine and leucine). Pepsin will not cleave bonds containing a valine, alanine, or glycine. Within the region of LT distal to the trypsin cleavage site at position 192 lies a potential pepsin cleavage site, a
5 leucine at position 211.

Using site directed mutagenesis, the leucine at position 211 of native LT and of LT(R192G) was replaced with an alanine residue, creating a single mutant LT(L211A) and the double mutant LT(R192G/L211A).

10 As shown in the Examples below (see section 6.2), the single mutant LT(L211A) retained the sensitivity to trypsin of native LT, while the double mutant LT(R192G/L211A) was insensitive to trypsin cleavage. This was expected since the single mutant LT(R192G) is not sensitive to trypsin cleavage. Unexpectedly, both the single and double mutants
15 of LT as well as native LT were found to be resistant to pepsin cleavage. Thus, even though the leucine residue at position 211 is a potential pepsin cleavage site, it does not appear to function as such.

LT(R192G) has substantially reduced enterotoxicity when compared to native LT in the patent mouse assay. 250 µg
20 of LT(R192G) has less enterotoxicity in the patent mouse model than does 5 µg of native LT. However, there is some amount of fluid accumulation. Surprisingly, even though the leucine residue at position 211 is not a pepsin cleavage site and LT(L211A) retains sensitivity to proteolytic cleavage by
25 trypsin, the single mutant LT(L211A) has greatly reduced enterotoxicity as well, exhibiting approximately the same level of enterotoxicity as LT(R192G). The double mutant LT(R192G/L211A) has essentially no enterotoxicity in this assay (see Figure 4).

30

5.1 LT(R192G/L211A) AND COMPOSITIONS THEREOF

The present invention encompasses a composition and methods for its use to promote the production of serum and/or mucosal antibodies as well as cell-mediated immune responses against antigens that are simultaneously administered with a genetically modified bacterial toxin.

The modified toxin is a form of the heat-labile enterotoxin (LT) of *E. coli* which through modification of the arginine at position 192 to glycine and the modification of the leucine at position 211 to arginine, has lost the trypsin sensitive site joining the A1 and A2 subunits, rendering the molecule non-toxic but still able to act as an immunological adjuvant. The invention is based on the discovery that LT(R192G/L211A) has utility as an adjuvant for mucosally or parenterally administered antigens; such administration results in the production of serum IgG and/or mucosal sIgA as well as cell-mediated immune responses against the antigens with which LT(R192G/L211A) is delivered.

5.2 PRODUCTION OF LT(R192G/L211A)

LT(R192G/L211A) can be produced by a number of means apparent to those of skill in the art. For example, plasmid pECD403, fully described in Example 6.1, can be utilized to produce substantially pure LT(R192G/L211A) in *E. coli*. LT(R192G/L211A) can be isolated by agarose affinity chromatography from bacteria expressing an LT(R192G/L211A) encoding plasmid. Alternate methods of purification will be apparent to those skilled in the art.

5.2.1 MODE OF ADMINISTRATION OF LT(R192G/L211A) AND UNRELATED ANTIGENS

In accordance with the present invention
LT(R192G/L211A) can be administered in conjunction with any
biologically relevant antigen and/or vaccine, such that an
5 increased immune response to said antigen and/or vaccine is
achieved. In one embodiment, LT(R192G/L211A) is a component
of a vaccine directed against an enterotoxigenic bacterial
organism.

In a preferred embodiment, the LT(R192G/L211A) and
10 antigen are administered simultaneously in a pharmaceutical
composition comprising an effective amount of LT(R192G/L211A)
and an effective amount of antigen. The mode of
administration is mucosal (i.e., intranasal, oral, rectal) or
parenteral (i.e., subcutaneous, intramuscular, intradermal,
15 intravenous, intraperitoneal).

In an alternate embodiment, the LT(R192G/L211A) and
antigen are administered separately within a short time of
each other.

The respective amounts of LT(R192G/L211A) and
antigen will vary depending upon the identity of the route of
20 administration, antigen employed and the species of animal to
be immunized. In one embodiment, the initial administration
of LT(R192G/L211A) and antigen is followed by a boost of the
relevant antigen. In another embodiment no boost is given.
The timing of boosting may vary, depending on the route,
25 antigen and the species being treated. The modifications in
route, dosage range and timing of boosting for any given
species and antigen are readily determinable by routine
experimentation. The boost may be of antigen alone or in
combination with LT(R192G/R211A).

30 The present invention also provides for a kit
useful in producing a protective immune response in a host to
a pathogen, said kit comprising an effective amount of

antigen and an adjuvant effective amount of a
LT(R192G/L211A).

5.3 ANTIGENS USEFUL IN THE INVENTION

5 The methods and compositions of the present
invention are intended for use both in immature and mature
vertebrates, in particular birds, mammals, and humans. Useful
antigens, as examples and not by way of limitation, would
include antigens from pathogenic strains of bacteria
(*Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Neisseria*
10 *gonorrhoea*, *Neisseria meningitidis*, *Corynebacterium*
diphtheriae, *Clostridium botulinum*, *Clostridium perfringens*,
Clostridium tetani, *Haemophilus influenzae*, *Klebsiella*
pneumoniae, *Klebsiella ozaenae*, *Klebsiella rhinoscleromatis*,
Staphylococcus aureus, *Vibrio cholerae*, *Escherichia coli*,
15 *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Aeromonas*
hydrophila, *Bacillus cereus*, *Edwardsiella tarda*, *Yersinia*
enterocolitica, *Yersinia pestis*, *Yersinia pseudotuberculosis*,
Shigella dysenteriae, *Shigella flexneri*, *Shigella sonnei*,
Salmonella typhimurium, *Treponema pallidum*, *Treponema*
20 *pertenue*, *Treponema carateneum*, *Borrelia vincentii*, *Borrelia*
burgdorferi, *Leptospira icterohemorrhagiae*, *Mycobacterium*
tuberculosis, *Toxoplasma gondii*, *Pneumocystis carinii*,
Francisella tularensis, *Brucella abortus*, *Brucella suis*,
Brucella melitensis, *Mycoplasma* spp., *Rickettsia prowazeki*,
25 *Rickettsia tsutsugumushi*, *Chlamydia* spp., *Helicobacter*
pylori); pathogenic fungi (*Coccidioides immitis*, *Aspergillus*
fumigatus, *Candida albicans*, *Blastomyces dermatitidis*,
Cryptococcus neoformans, *Histoplasma capsulatum*); protozoa
(*Entamoeba histolytica*, *Trichomonas tenax*, *Trichomonas*
30 *hominis*, *Trichomonas vaginalis*, *Trypanosoma gambiense*,
Trypanosoma rhodesiense, *Trypanosoma cruzi*, *Leishmania*
donovani, *Leishmania tropica*, *Leishmania braziliensis*,

Pneumocystis pneumonia, *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malaria*); or Helminths (*Enterobius vermicularis*, *Trichuris trichiura*, *Ascaris lumbricoides*, *Trichinella spiralis*, *Strongyloides stercoralis*, *Schistosoma japonicum*, *Schistosoma mansoni*, *Schistosoma haematobium*, and hookworms) either presented to the immune system in whole cell form or in part isolated from media cultures designed to grow said organisms which are well known in the art, or protective antigens from said organisms obtained by genetic engineering techniques or by chemical synthesis.

Other relevant antigens would be pathogenic viruses (as examples and not by limitation: Poxviridae, Herpesviridae, Herpes Simplex virus 1, Herpes Simplex virus 2, Adenoviridae, Papovaviridae, Enteroviridae, Picornaviridae, Parvoviridae, Reoviridae, Retroviridae, influenza viruses, parainfluenza viruses, mumps, measles, respiratory syncytial virus, rubella, Arboviridae, Rhabdoviridae, Arenaviridae, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis E virus, Non-A/Non-B Hepatitis virus, Rhinoviridae, Coronaviridae, Rotoviridae, and Human Immunodeficiency Virus) either presented to the immune system in whole or in part isolated from media cultures designed to grow such viruses which are well known in the art or protective antigens therefrom obtained by genetic engineering techniques or by chemical synthesis.

Further examples of relevant antigens include, but are not limited to, vaccines. Examples of such vaccines include, but are not limited to, influenza vaccine, pertussis vaccine, diphtheria and tetanus toxoid combined with pertussis vaccine, hepatitis A vaccine, hepatitis B vaccine, hepatitis C vaccine, hepatitis E vaccine, Japanese encephalitis vaccine, herpes vaccine, measles vaccine, rubella vaccine, mumps vaccine, mixed vaccine of measles, mumps and rubella, papillomavirus vaccine, parvovirus

vaccine, respiratory syncytial virus vaccine, Lyme disease vaccine, polio vaccine, varicella vaccine, gonorrhea vaccine, schistosomiasis vaccine, rotavirus vaccine, mycoplasma vaccine pneumococcal vaccine, meningococcal vaccine, campylobacter vaccine, helicobacter vaccine, cholera vaccine, enterotoxigenic *E. coli* vaccine, enterohemorrhagic *E. coli* vaccine, shigella vaccine, salmonella vaccine and others. These can be produced by known common processes. In general, such vaccines comprise either the entire organism or virus grown and isolated by techniques well known to the skilled artisan or comprise relevant antigens of these organisms or viruses which are produced by genetic engineering techniques or chemical synthesis. Their production is illustrated by, but not limited to, as follows:

Influenza vaccine: a vaccine comprising the whole or part of hemagglutinin, neuraminidase, nucleoprotein and matrix protein which are obtainable by purifying a virus, which is grown in embryonated eggs, with ether and detergent, or by genetic engineering techniques or chemical synthesis.

Pertussis vaccine: a vaccine comprising the whole or a part of pertussis toxin, hemagglutinin and K-agglutinin which are obtained from avirulent toxin with formalin which is extracted by salting-out or ultracentrifugation from the culture broth or bacterial cells of *Bordetella pertussis*, or by genetic engineering techniques or chemical synthesis.

Diphtheria and tetanus toxoid combined with pertussis vaccine: a vaccine mixed with pertussis vaccine, diphtheria and tetanus toxoid.

Japanese encephalitis vaccine: a vaccine comprising the whole or part of an antigenic protein which is obtained by culturing a virus intracerebrally in mice and purifying the virus particles by centrifugation or ethyl alcohol and inactivating the same, or by genetic engineering techniques or chemical synthesis.

Hepatitis B vaccine: a vaccine comprising the whole or part of an antigen protein which is obtained by isolating and purifying the HBs antigen by salting-out or ultracentrifugation, obtained from hepatitis carrying blood,
5 or by genetic engineering techniques or by chemical synthesis.

Measles vaccine: a vaccine comprising the whole or part of a virus grown in a cultured chick embryo cells or embryonated egg, or a protective antigen obtained by genetic engineering or chemical synthesis.

10 Rubella vaccine: a vaccine comprising the whole or part of a virus grown in cultured chick embryo cells or embryonated egg, or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

Mumps vaccine: a vaccine comprising the whole or
15 part of a virus grown in cultured rabbit cells or embryonated egg, or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

Mixed vaccine of measles, rubella and mumps: a vaccine produced by mixing measles, rubella and mumps
20 vaccines.

Rotavirus vaccine: a vaccine comprising the whole or part of a virus grown in cultured MA 104 cells or isolated from the patient's feces, or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

25 Mycoplasma vaccine: a vaccine comprising the whole or part of mycoplasma cells grown in a liquid culture medium for mycoplasma or a protective antigen obtained by genetic engineering techniques or chemical synthesis.

Those conditions for which effective prevention may be achieved by the present method will be obvious to the
30 skilled artisan.

The vaccine preparation compositions of the present invention can be prepared by mixing the above illustrated

antigens and/or vaccines with LT(R192G/L211A) at a desired ratio. Pyrogens or allergens should naturally be removed as completely as possible. The antigen preparation of the present invention can be used by preparing the antigen per se and the LT(R192G/L211A) separately or together.

Further, the present invention encompasses a kit comprising an effective amount of antigen and an adjuvant effective amount of LT(R192G/L211A). In use, the components of the kit can either first be mixed together and then administered or the components can be administered separately within a short time of each other.

The vaccine preparation compositions of the present invention can be combined with either a liquid or solid pharmaceutical carrier, and the compositions can be in the form of tablets, capsules, powders, granules, suspensions or solutions. The compositions can also contain suitable preservatives, coloring and flavoring agents, or agents that produce slow release. Potential carriers that can be used in the preparation of the pharmaceutical compositions of this invention include, but are not limited to, gelatin capsules, sugars, cellulose derivations such as sodium carboxymethyl cellulose, gelatin, talc, magnesium stearate, vegetable oil such as peanut oil, etc., glycerin, sorbitol, agar and water. Carriers may also serve as a binder to facilitate tableting of the compositions for convenient administration.

25

6. EXAMPLES

The following examples are presented for purposes of illustration only and are not intended to limit the scope of the invention in any way.

30

6.1. CONSTRUCTION OF LT(R192G/L211A)

The wild-type LT toxin is encoded on a naturally occurring plasmid found in strains of enterotoxigenic *E. coli*

capable of producing this toxin. Clements et al. had previously cloned the LT gene from a human isolate of *E. coli* designated H10407. This subclone consists of a 5.2 kb DNA fragment from the enterotoxin plasmid of H10407 inserted into the *Pst*I site of plasmid pBR322 (Clements et al, 1983, *Infect. Immun.* 40:653). This recombinant plasmid, designated pDF82, has been extensively characterized and expresses LT under control of the native LT promoter. From pDF82, Clements et al. derived plasmid pBD95, which is fully described in PCT Publication WO96/06627.

Figure 1A shows the construction of plasmid pCS95, which was constructed by inserting the native LT-A subunit regulatory region upstream from the LT-A coding region of pBD95. Figure 1B shows the Arg to Gly mutation at position 192. The *Bam*HI and *Xba*I restriction sites referred to in the diagram as "new" were added by site directed mutagenesis, as described in PCT Publication WO96/06627. The new *Xba*I site was added through a silent mutation, resulting in no alteration of the amino acid sequence of the peptide encoded by the gene.

Then, starting with the single mutant plasmid pCS95, Ala was substituted for Leu at position 211 (i.e., GCC = Ala replaces CTC = Leu) by site directed mutagenesis. The resultant mutant LT was then purified by agarose affinity chromatography from bacteria expressing the mutated plasmid, named pECD403, which had been confirmed by sequencing.

This mutant LT, designated LT(R192G/L211A) was then examined by SDS-polyacrylamide gel electrophoresis for modification of the trypsin sensitive bond. Samples were examined with and without exposure to trypsin and compared with native (unmodified) LT. LT(R192G/L211A) does not dissociate into A₁ and A₂ when incubated with trypsin, thereby indicating that sensitivity to protease has been removed.

6.2. LT(R192G/L211A) EXHIBITS RESISTANCE TO PEPSIN CLEAVAGE

Using site directed mutagenesis, the leucine at position 211 of native LT and of LT(R192G) was replaced with an alanine residue, creating a single mutant LT(L211A) and
5 the double mutant LT(R192G/L211A).

Following purification by galactose affinity chromatography, both of these constructs were examined by SDS-PAGE analysis. As shown in Figure 3, the single mutant LT(L211A) retained the sensitivity to trypsin of native LT
10 (compare upper panel lanes 1 and 2 with lanes 6 and 7) while the double mutant LT(R192G/L211A) was insensitive to trypsin cleavage (upper panel lanes 8 and 9). This was expected since the single mutant LT(R192G) is not sensitive to trypsin cleavage (upper panel lanes 4 and 5). Unexpectedly, both the
15 single and double mutants of LT as well as native LT were found to be resistant to pepsin cleavage. Thus, even though the leucine residue at position 211 is a potential pepsin cleavage site, it does not appear to function as such.

6.3. ENTEROTOXICITY IN THE PATENT MOUSE ASSAY

20 LT(R192G/L211A) and LT(L211A) were examined for enterotoxicity in the patent mouse assay. As can be seen in Figure 4, LT(R192G) has substantially reduced enterotoxicity when compared to native LT in this assay. 250 µg of LT(R192G) has less enterotoxicity in the patent mouse model than does 5 µg of native LT. However, there is some small
25 amount of fluid accumulation. Surprisingly, even though the leucine residue at position 211 is not a pepsin cleavage site and LT(L211A) retains sensitivity to proteolytic cleavage by trypsin, the single mutant LT(L211A) has greatly reduced enterotoxicity as well and approximately the same level of
30 enterotoxicity as LT(R192G). The double mutant LT(R192G/L211A) has essentially no enterotoxicity in this assay.

6.4. INTRANASAL ADJUVANTICITY OF LT(R192G/L211A)

Ovalbumin (Ova) was selected as a representative antigen for these studies. A number of investigations, including our own (Clements et al., 1988, *Vaccine* 6:269-277; Dickinson and Clements, 1996, *Mucosal Vaccines* :73-87; Dickinson and Clements, 1995, *Infection and Immunity*, 63:1617-1623; Tsuji et al., 1997, *Immunology* 90:176-182; Yamamoto et al., 1997, *Journal of Experimental Medicine* 185:1203-1210; Yamamoto et al., 1997, *Proceedings of the National Academy of Sciences* 94:5267-5272; DiTommaso et al., 1996, *Infection and Immunity* 64:974-979; Douce et al., 1995, *Proceedings of the National Academy of Sciences* 92:1644-1648; Douce et al., 1997, *Infection and Immunity* 65:2821-2828), have used this protein and it provides a useful and necessary reference for comparison to other studies. For those studies, both serum anti-Ova IgG and Ova-specific T-cell responses were examined.

Mice were immunized intranasally with Ova alone or in conjunction with 5 μ g of LT(R192G) or LT(R192G/L211A). Serum anti-Ova IgG was determined by ELISA. There were seven animals per group and the means for each data point are shown. As shown in Figure 5, mice immunized intranasally with Ova in conjunction with LT(R192G) had serum anti-Ova IgG responses indistinguishable from animals immunized with Ova in conjunction with LT(R192G/L211A). When the Ova-specific T-cell responses from these animals were examined, both the Th2/IL-10 (Figure 6) and the Th1/IFN-gamma (Figure 7) anti-Ova responses were equivalent when either LT(R192G) or LT(R192G/L211A) was used in the adjuvant formulation. There is no significant difference in the IFN-gamma and IL-10 responses between these two groups.

7. DEPOSIT OF MICROORGANISMS

The following microorganism containing the designated plasmid was deposited with the American Type
5 Culture Collection (ATCC), (present address: 1081 University Boulevard, Manassas, VA 20110-2209) on March 13, 1998, and has been assigned the indicated accession number:

	<u>Microorganism</u>	<u>Accession Number</u>
10	E. coli JM83 pECD403	98697

Although the invention is described in detail with reference to specific embodiments thereof, it will be understood that variations which are functionally equivalent are within the scope of this invention. Indeed, various
15 modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

20 Various publications are cited herein, the disclosure of which are incorporated by reference in their entireties.

25

30

MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page <u>26</u> , lines <u>8-9</u> of the description *	
A. IDENTIFICATION OF DEPOSIT *	
Further deposits are identified on an additional sheet *	
Name of depositary institution *	
American Type Culture Collection	
Address of depositary institution (including postal code and country) *	
10801 University Blvd. Manassas, VA 20110-2209 US	
Date of deposit * <u>March 13, 1998</u> Accession Number * <u>98697</u>	
B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (if the indications are not all designated States)	
D. SEPARATE FURNISHING OF INDICATIONS * (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input checked="" type="checkbox"/> This sheet was received with the International application when filed (to be checked by the receiving Office) <div style="text-align: right;"> Paralegal Specialist IAPD-PCT Operations <u>17031405-3745</u> (Authorized Officer) </div>	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau * <div style="display: flex; justify-content: space-between; margin-top: 20px;"> <div>was</div> <div>_____ (Authorized Officer)</div> </div>	

Form PCT/RO/134 (January 1981)

WHAT IS CLAIMED IS:

1. A composition comprising a mutant *E. coli* heat-labile enterotoxin holotoxin, in which arginine at amino acid position 192 is replaced with glycine and in which leucine at amino acid position 211 is replaced with alanine, which holotoxin has immunologic adjuvant activity and is:
 - (a) substantially less toxic than native *E. coli* heat-labile enterotoxin holotoxin as measured in the Y-1 adrenal cell assay,
 - (b) substantially less toxic than native *E. coli* heat-labile enterotoxin holotoxin as measured in the patent mouse assay, and
 - (c) less toxic than a composition consisting of isolated LT(R192G) as measured in the patent mouse assay.
2. The composition of claim 1 in which the holotoxin is encoded by the plasmid having ATCC Accession number XXX, which expresses both subunit A and subunit B of the *E. coli* heat-labile enterotoxin.
3. A preparation comprising an antigen in combination with the composition according to claim 1.
4. The preparation according to claim 3, in which the antigen is selected from the group of antigens consisting of bacterial, fungal, protozoal viral, helmenthal and other microbial pathogenic antigens.
5. The preparation according to claim 4, in which the antigen is selected from the group consisting of *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Neisseria gonorrhoea*, *Neisseria meningitidis*, *Corynebacterium*

diphtheriae, *Clostridium botulinum*, *Clostridium perfringens*,
Clostridium tetani, *Haemophilus influenzae*, *Klebsiella*
pneumoniae, *Klebsiella ozaenae*, *Klebsiella rhinoscleromatis*,
Staphylococcus aureus, *Vibrio cholerae*, *Escherichia coli*,
5 *Pseudomonas aeruginosa*, *Campylobacter* (*Vibrio*) *fetus*,
Campylobacter jejuni, *Aeromonas hydrophila*, *Bacillus cereus*,
Edwardsiella tarda, *Yersinia enterocolitica*, *Yersinia pestis*,
Yersinia pseudotuberculosis, *Shigella dysenteriae*, *Shigella*
flexneri, *Shigella sonnei*, *Salmonella typhimurium*, *Treponema*
10 *pallidum*, *Treponema pertenue*, *Treponema carateneum*, *Borrelia*
vincentii, *Leptospira icterohemorrhagiae*, *Mycobacterium*
tuberculosis, *Toxoplasma gondii*, *Pneumocystis carinii*,
Francisella tularensis, *Brucella abortus*, *Brucella suis*,
Brucella melitensis, *Mycoplasma* spp., *Rickettsia prowazeki*,
Rickettsia tsutsugumushi, *Chlamydia* spp., *Helicobacter*
15 *pylori*, *Coccidioides immitis*, *Aspergillus fumigatus*, *Candida*
albicans, *Blastomyces dermatitidis*, *Cryptococcus neoformans*,
Histoplasma capsulatum, *Entamoeba histolytica*, *Trichomonas*
tenax, *Trichomonas hominis*, *Trichomonas vaginalis*,
Trypanosoma gambiense, *Trypanosoma rhodesiense*, *Trypanosoma*
20 *cruzi*, *Leishmania donovani*, *Leishmania tropica*, *Leishmania*
braziliensis, *Pneumocystis pneumonia*, *Enterobius*
vermicularis, *Trichuris trichiura*, *Ascaris lumbricoides*,
Trichinella spiralis, *Strongyloides stercoralis*, *Schistosoma*
japonicum, *Schistosoma mansoni*, *Schistosoma haematobium*,
25 *variola virus*, *vaccinia virus*, *cowpox virus*, *varicella-zoster*
virus, *Herpes Simplex virus 1*, *Herpes Simplex virus 2*,
influenza viruses, *parainfluenza virus*, *mumps*, *measles*,
respiratory syncytial virus, *rubella*, *Hepatitis A virus*,
Hepatitis B virus, *Hepatitis C virus*, *Hepatitis E virus*, and
30 *Non-A/Non-B Hepatitis virus antigens*.

6. A composition useful in producing a protective immune response to a pathogen in a host comprising an admixture of an effective amount of an antigen and an adjuvant effective amount of the composition according to
5 claim 1.

7. A kit useful in producing a protective immune response in a host to a pathogen comprising two components:
(a) an effective amount of antigen and (b) an adjuvant effective amount of a mutant *E. coli* heat-labile enterotoxin
10 holotoxin, in which arginine at amino acid position 192 is replaced with glycine and in which leucine at amino acid position 211 is replaced with alanine, which holotoxin has immunologic adjuvant activity and is:

- 15 (a) substantially less toxic than native *E. coli* heat-labile enterotoxin holotoxin as measured in the Y-1 adrenal cell assay,
- (b) substantially less toxic than native *E. coli* heat-labile enterotoxin holotoxin as measured in the patent mouse assay, and
- 20 (c) less toxic than a composition consisting of isolated LT(R192G) as measured in the patent mouse cell assay.

8. A method of creating or sustaining a protective or adaptive immune response to an antigen in a host
25 comprising orally administering an admixture of an effective amount of an antigen and an adjuvant effective amount of a mutant *E. coli* heat-labile enterotoxin holotoxin, in which arginine at amino acid position 192 is replaced with glycine and in which leucine at amino acid position 211 is replaced
30 with alanine, which holotoxin is substantially less toxic than native *E. coli* heat-labile enterotoxin holotoxin as measured in the Y-1 adrenal cell assay, substantially less

toxic than native *E. coli* heat-labile enterotoxin holotoxin as measured in the patent mouse assay, and less toxic than compositions of isolated LT(R192G) as measured in the patent mouse assay, in an orally acceptable pharmaceutical carrier.

5

9. The method of claim 8 where a serum response is produced.

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10. The method of claim 8 where a cell-mediated immune response is produced.

11. The method of claim 8 where a mucosal response is produced.

12. The method of claim 8 further comprising
15 administering a subsequent boost of the antigen.

13. The method of claim 8 wherein the antigen is derived from the group consisting of bacteria, viruses, protozoa, fungi, helminths, and other microbial pathogens.

20

14. A method of inducing a protective immune response to an antigen in a host comprising orally administering an effective amount of an antigen in conjunction with an adjuvant effective amount of a mutant *E. coli* heat-labile enterotoxin holotoxin, in which arginine at
25 amino acid position 192 is replaced with glycine and in which leucine at amino acid position 211 is replaced with alanine, which holotoxin has immunologic adjuvant activity and is:

(a) substantially less toxic than native *E. coli* heat-labile enterotoxin holotoxin as measured in the Y-1
30 adrenal cell assay,

- (b) substantially less toxic than native *E. coli* heat-labile enterotoxin holotoxin as measured in the patent mouse assay, and
- 5 (c) less toxic than a composition consisting of isolated LT(R192G) as measured in the patent mouse assay.

15. The method of claim 14 in which the antigen and the holotoxin are administered simultaneously.

- 10 16. The method of claim 14 in which the antigen and holotoxin are administered separately within a short time of each other.

17. A method of inducing a protective immune
15 response against an enterotoxic bacterial organism comprising administering a mutant *E. coli* heat-labile enterotoxin holotoxin, in which arginine at amino acid position 192 is replaced with glycine and in which leucine at amino acid position 211 is replaced with alanine, which holotoxin has
20 immunologic adjuvant activity and is:

- (a) substantially less toxic than native *E. coli* heat-labile enterotoxin holotoxin as measured in the Y-1 adrenal cell assay, and
- (b) less toxic than a composition consisting of
25 isolated LT(R192G) as measured in the patent mouse assay,
as a component of a vaccine directed against the enterotoxic bacterial organism.

18. The method of claim 17 wherein the enterotoxic
30 bacterial organism is selected from the group consisting of enterotoxic bacterial organisms which express a cholera-like toxin.

19. The method of claim 17 wherein the enterotoxigenic bacterial organism is selected from the group consisting of *Escherichia* spp. and *Vibrio* spp.

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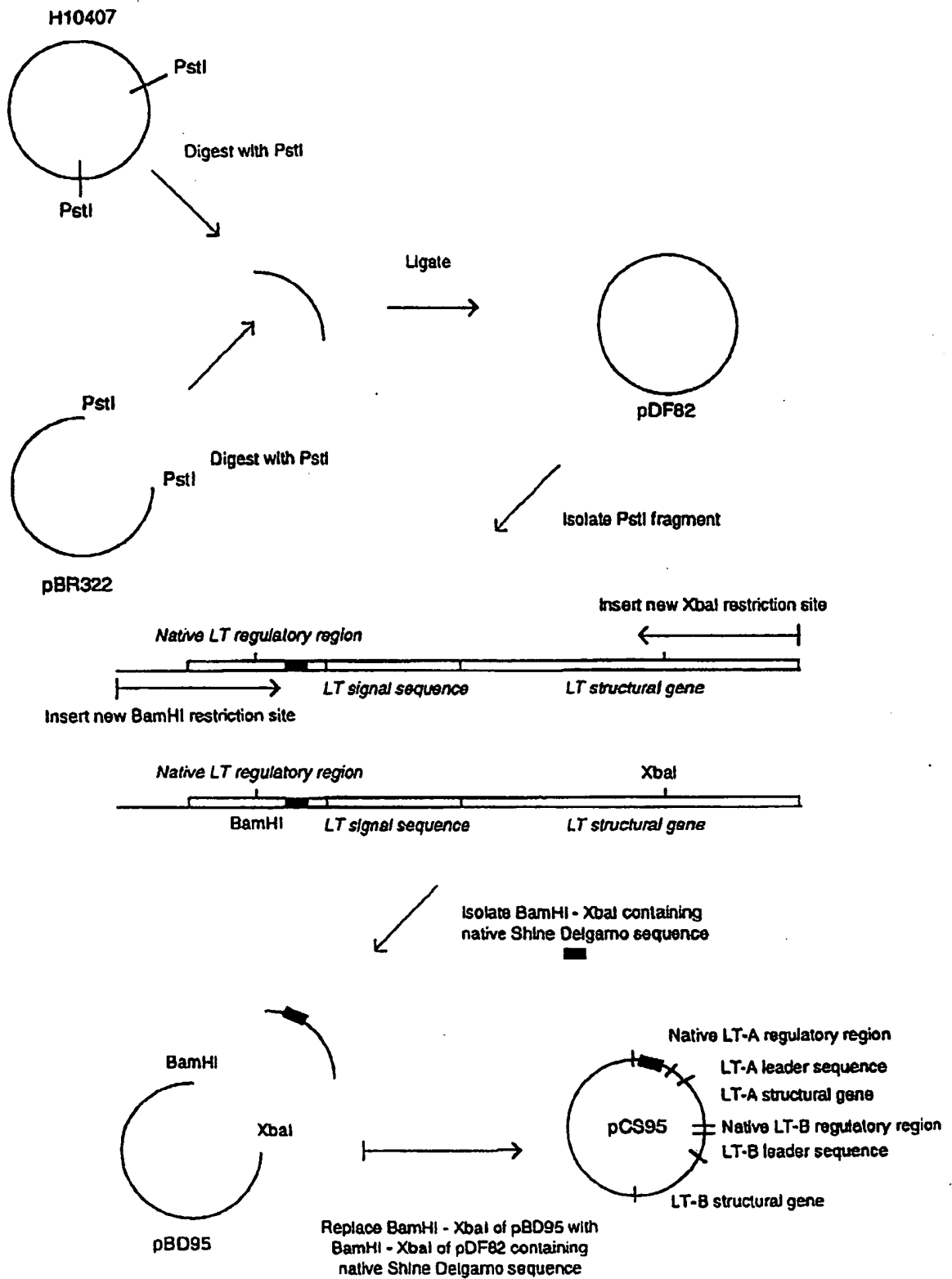


Figure 1A

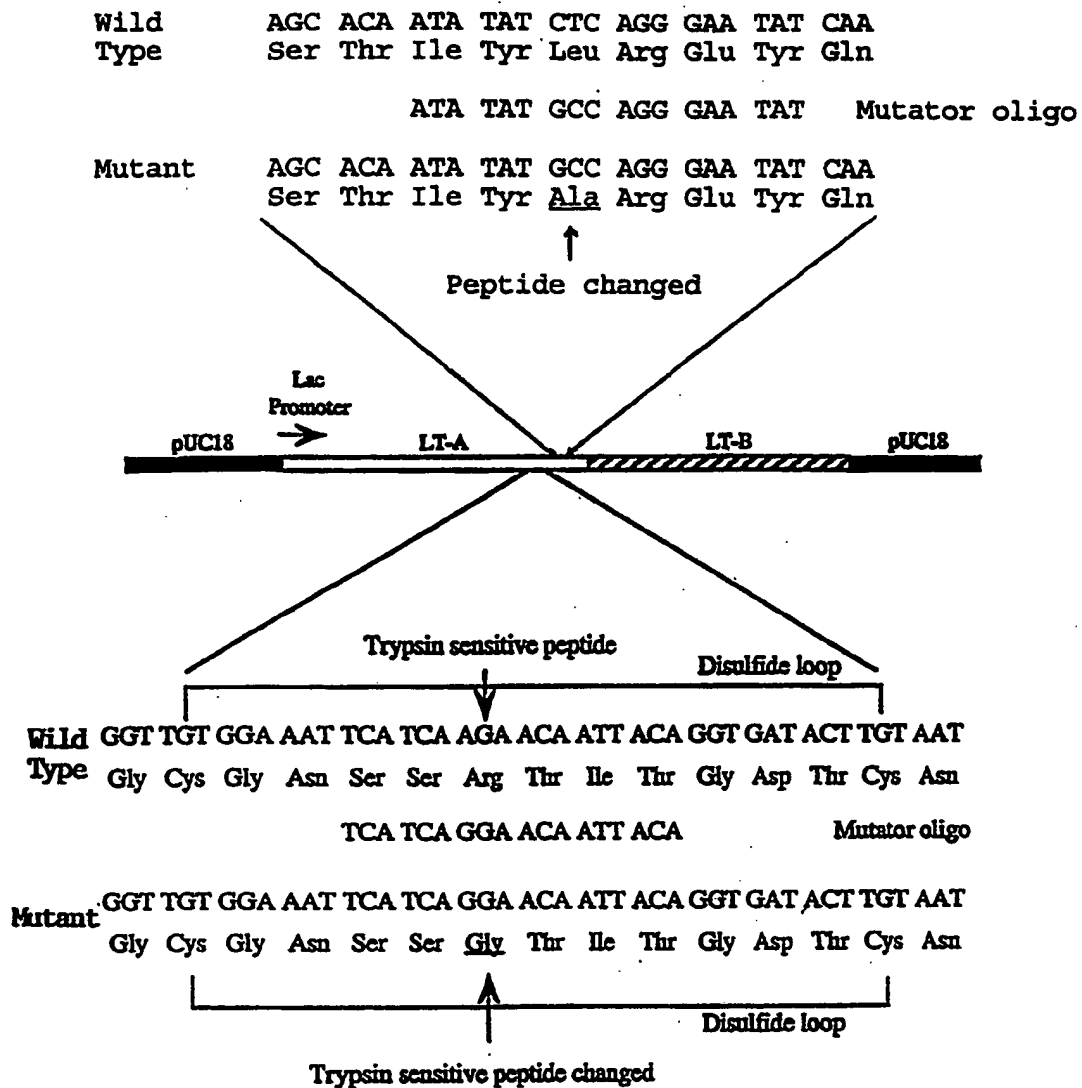


Figure 1B

187

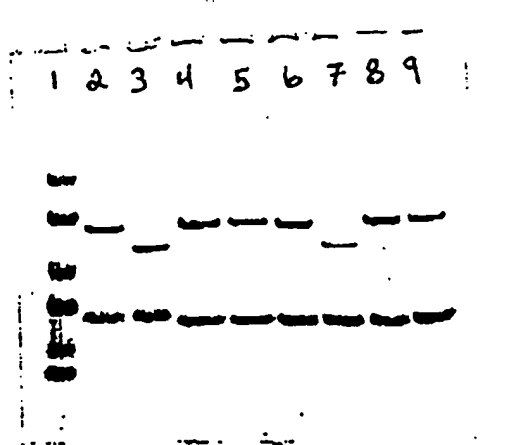
192

TGT GGA AAT TCA TCA AGA ACA ATT ACA GGT GAT ACT TGT AAT
Cys Gly Asn Ser Ser Arg Thr Ile Thr Gly Asp Thr Cys Asn

GAG GAG ACC CAG AAT CTG AGC ACA ATA TAT ²¹¹CTC AGG AAA ²¹⁴TAT
Glu Glu Thr Gln Asn Leu Ser Thr Ile Tyr Leu Arg Lys Tyr

Figure 2

Lane	Sample
1	Marker
2	LT
3	LT + Trypsin
4	LT(R192G)
5	LT(R192G) + Trypsin
6	LT(211A)
7	LT(211A) + Trypsin
8	LT(R192G/L211A)
9	LT(R192G/L211A) + Trypsin



Lane	Sample
1	Marker
2	LT
3	LT + Pepsin
4	LT(R192G)
5	LT(R192G) + Pepsin
6	LT(211A)
7	LT(211A) + Pepsin
8	LT(R192G/L211A)
9	LT(R192G/L211A) + Pepsin

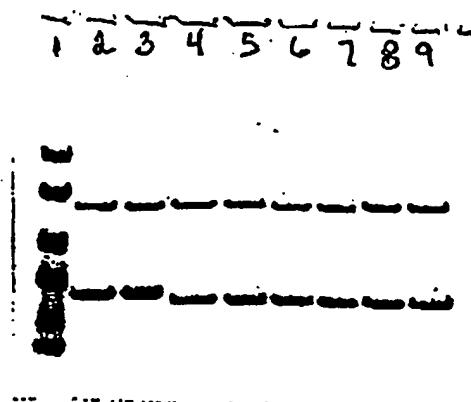
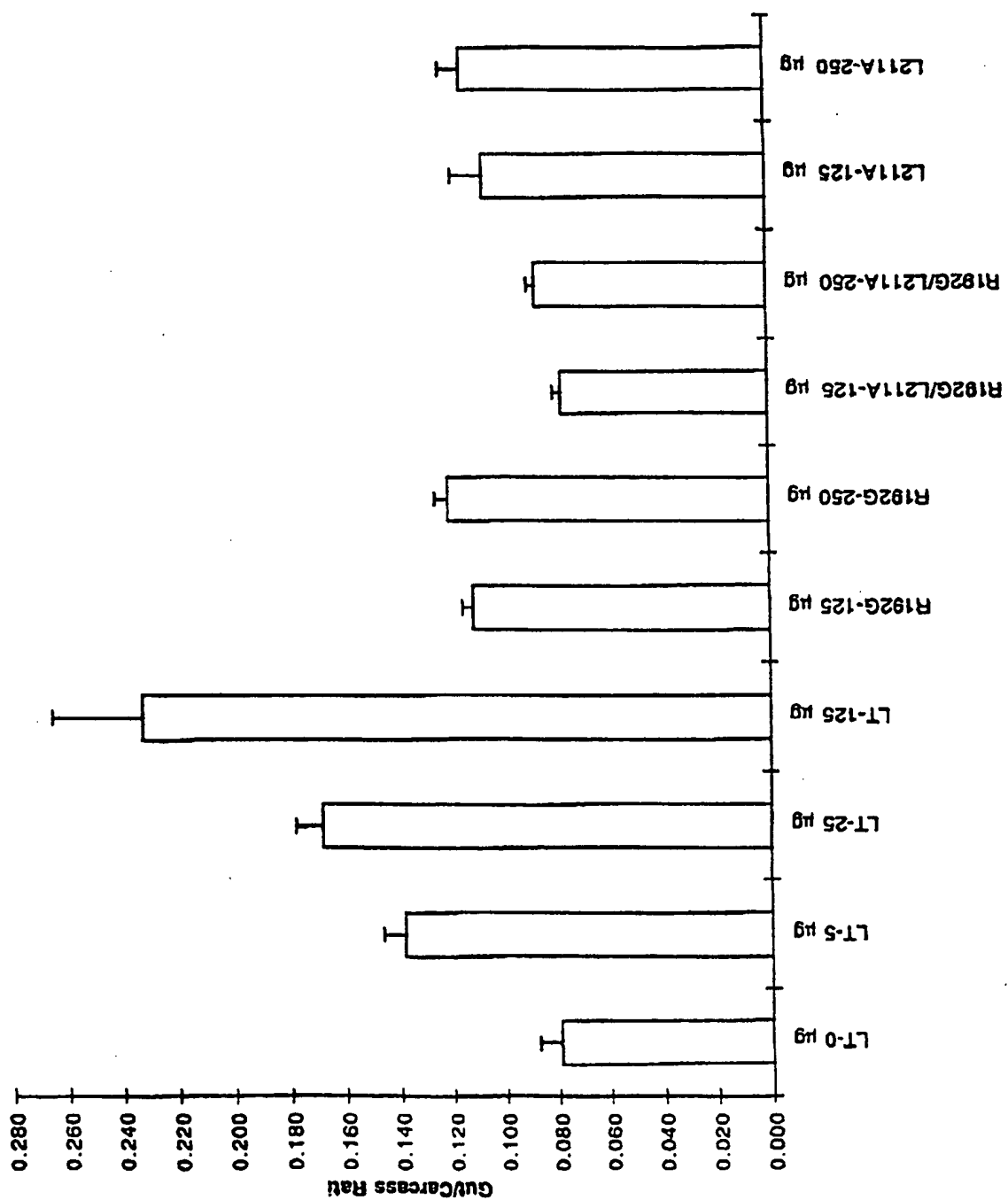


Figure 3

Figure 4



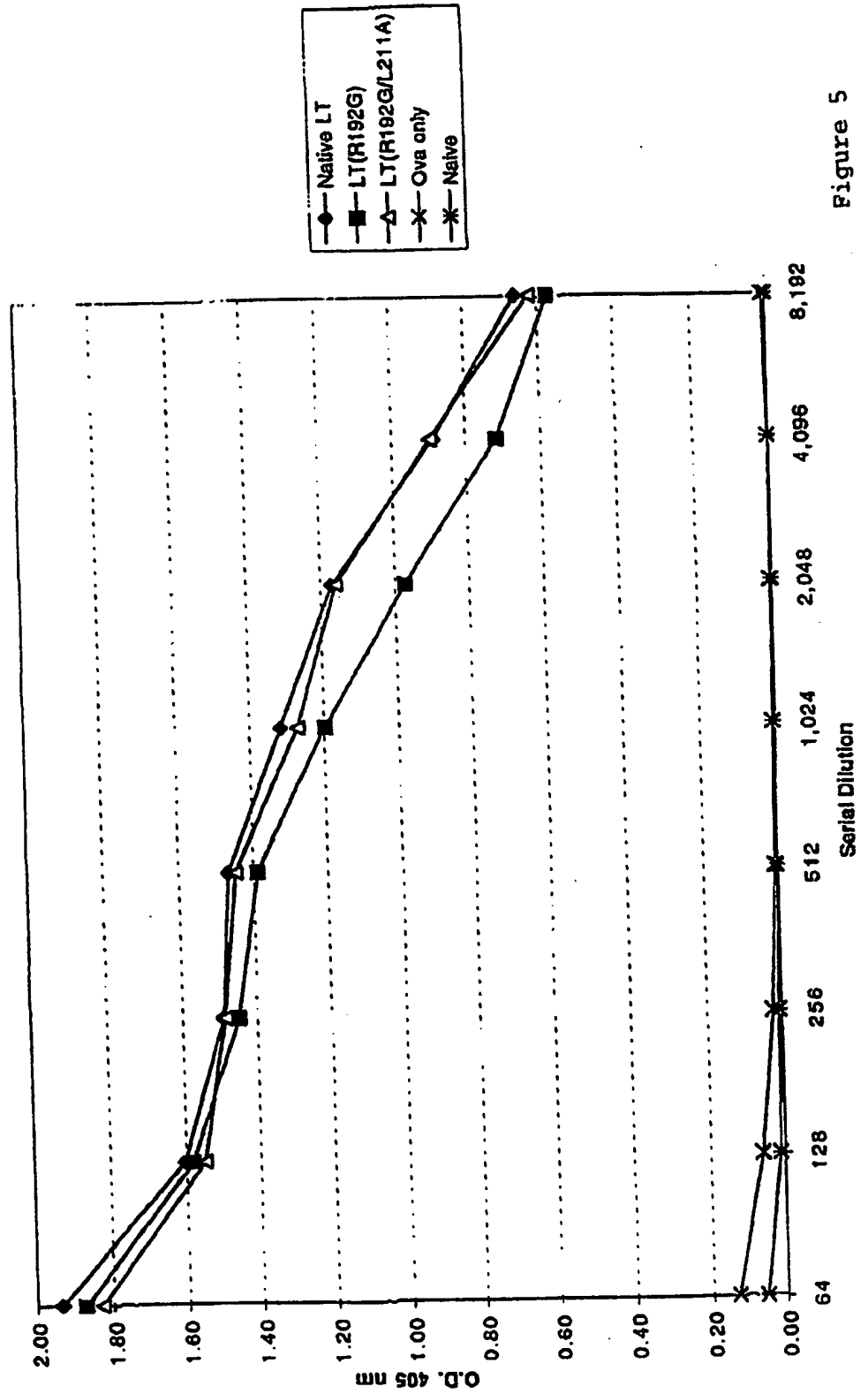


Figure 5

Figure 6

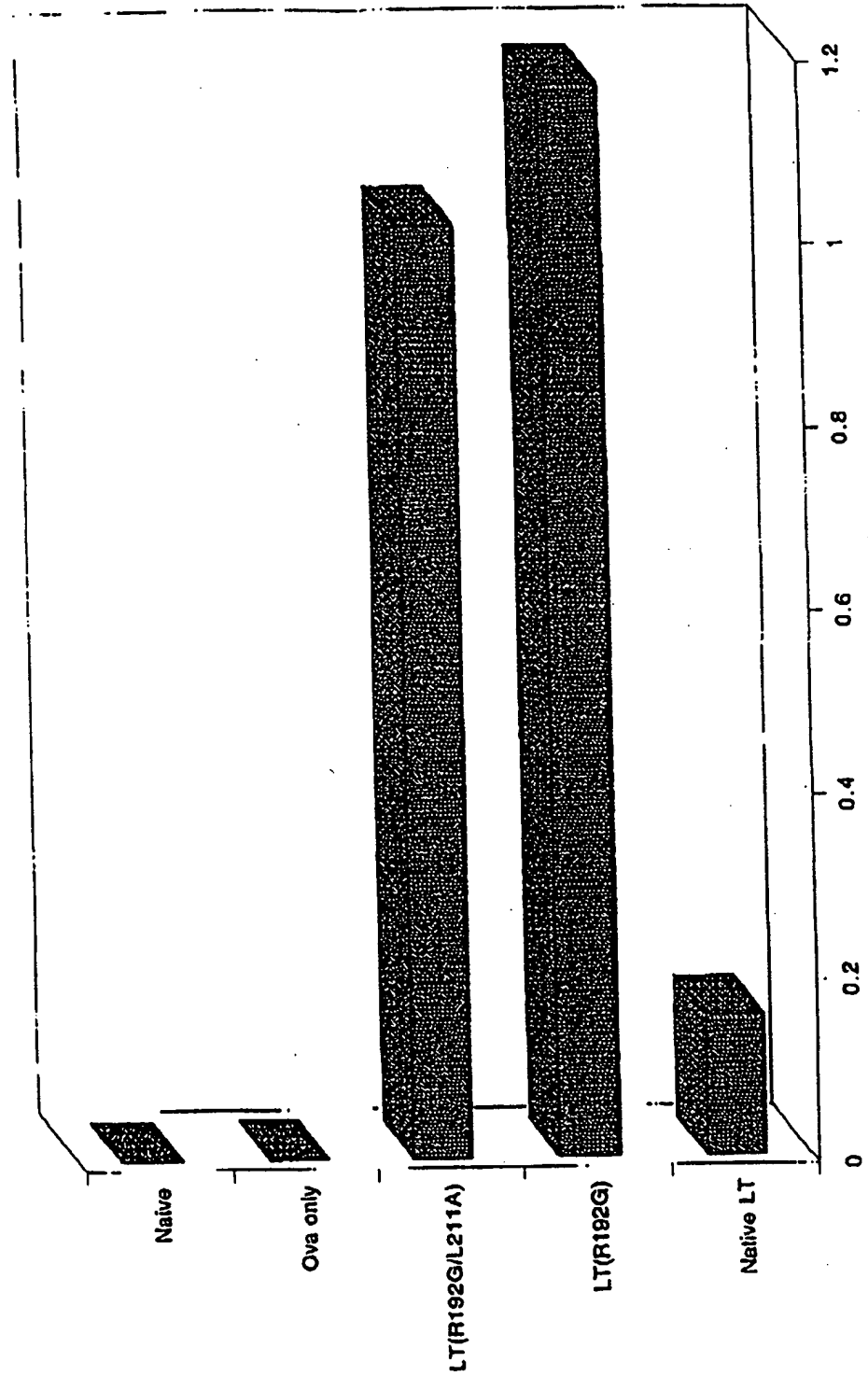
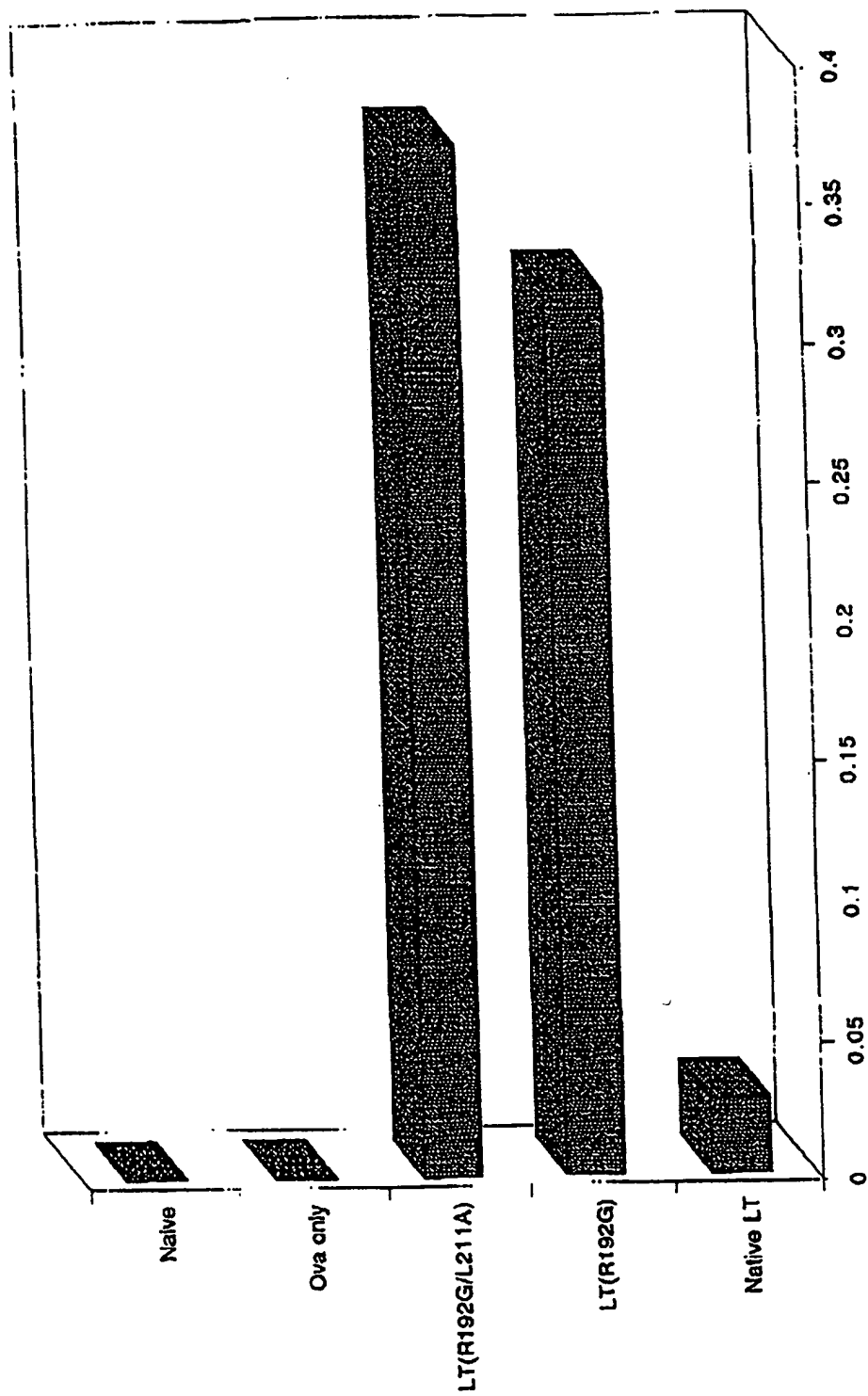


Figure 7



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05623**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 39/295, 39/116, 45/00, 39/00, 39/02, 39/108; G01N 33/53

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/201.1, 203.1, 202.1, 278.1, 184.1, 236.1, 241.1, 257.1, 282.1, 832; 435/975

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, EMBASE, APS, INSIDE CONFERENCES, FEDRIP, PASCAL, JAPIO, JICSTEPLUS, CABA, AGRICOLA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SIXMA et al. Refined structure of Escherichia coli heat-labile enterotoxin, a close relative of cholera toxin. J. Mol. Biol. 1993, Vol. 30, pages 890-918, see entire abstract.	1-19
A	WO 96/06627 A1 (THE ADMINISTRATORS OF THE TULANE EDUCATIONAL FUND) 07 March 1996, see entire document.	1-19
A	WO 92/19265 A1 (AMGEN INC.), see entire document.	1-19
A	DOUCE et al. Mutants of Escherichia coli heat-labile toxin ADP-ribosyltransferase activity act as non-toxic, mucosal adjuvants. Proc. Nat. Acad. Sci., USA. February 1995, Vol. 9, No. 5, pages 1644-1648, see entire document.	1-19

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
24 MAY 1999Date of mailing of the international search report
29 JUN 1999Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05623

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GRANT et al. Role of trypsin-like cleavage at arginine 192 in the enzymatic and cytotoxic activities of Escherichia coli heat-labile enterotoxin. Infect. Immun. October 1994, Vol. 62, No. 10, pages 4270-4278, see entire document.	1-19

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/05623

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/201.1, 203.1, 202.1, 278.1, 184.1, 236.1, 241.1, 257.1, 282.1, 832; 435/975